Methods

Thermal-visual place learning arena

To control the thermal landscape, we developed an array of sixty-four 1 inch-square individually addressable thermoelectric modules (TEM tiles) arranged in an 8x8 grid (Oven Industries, Mechanicsburg, PA) (Fig. 1b). This array forms the floor of our test arena and is covered with black masking tape to create a uniform, featureless surface that can be replaced between experiments. Importantly, no thermal gradients exist that could guide flies to the cool spot from a distance (see Fig. 1b and Supplementary Fig. 7), as characterized by thermal imaging (Optotherm, Sewickley, PA) and thermocouple measurements. Additionally, the absence of place learning in the dark and uncoupled condition (evidenced by near-zero Direction and Probe learning indices, Fig. 2c and Fig. 3c) confirms that there are no significant non-visual or idiothetic cues in the arena that guide flies to the cool spot.

To confine flies to this surface, a 3mm high, 8 inch diameter aluminum ring was placed around the outer perimeter of the arena and covered with a glass disk coated with a slippery silicon film (Sigmacote, Sigma-Aldrich). To keep flies from walking on the walls, the aluminum ring was heated to $>50^{o}$C using insulated resistance wire (Pelican Wire, 29 AWG Nichrome 60

w/Kapton). Peripheral visual cues were provided using an electronically controlled LED display positioned around the outer perimeter of the arena[29] (Fig. 1a). In visual place learning trials, the LED panels were set to display a visual landscape composed of evenly spaced vertical, horizontal, and diagonal bars. When viewed from the arena's center, the width of each bar covered 15 degrees of the LED display. When viewed from a distance of 8.8 inches, the maximum distance possible in our arena, each visual element subtends ~8° of the fly's visual field and should be easily resolvable by the fly. The entire arena is illuminated with infrared light (Smart Vision Lights, Muskegon, MI) and fly activity was recorded with a Basler (Ahrensburg, Germany) 622f CMOS camera fitted with an infrared passing filter.

Visual place learning protocol and analysis

The experimental protocol included 10 training trials (5 minutes each) followed by a probe trial (trial 11) where the visual display was relocated in the absence of a cool spot. At the end of the experiment, flies were tested in a temperature preference trial[32, 33] and an optomotor trial[34] to measure normal thermal and visual responses. Flies were tracked off-line using Ctrax fly tracking software[30]. Fly centroid data were imported into MATLAB (Mathworks, Natick, MA) and processed using custom scripts. In Fig. 2, Time to target, Path length, and Velocity are calculated, per fly, for the time window from the start of the trial until each fly reaches the target tile. Direction index (quadrant choice) is calculated as the number of flies that first enter the quadrant containing the cool tile (# correct) minus the number of flies that first pass into the opposite quadrant (# incorrect) divided by the total number of flies. To test if flies exhibit a bias for certain quadrants or rotation directions during training in the visual place learning arena, we tested for the dependence of the time to target on the target quadrant and on the rotation direction

(CW or CCW). This was accomplished by calculating the difference between the time to target for each trial (from each experiment) and the mean time to target for each trial across all experiments. The differences from mean (in seconds) for training to quadrant 1, 2, 3, or 4 are 1.7 ± 2.3, -0.5 ± 2.7, 0.0 ± 2.2, and -1.2 ± 2.2 seconds respectively. The differences from mean (in seconds) for clockwise and counterclockwise rotations are 0.6 ± 1.5 and -0.6 ± 1.8 seconds respectively. Error is reported as SEM. For both tests, there are no statistically significant comparisons using one-way ANOVA at $p<0.05$.

In Fig. 3b, percentage of time spent in each of the quadrants was tested for statistical significance using one-way ANOVA with a Bonferroni correction for multiple comparisons. Flies spend significantly more time searching in Q2 when compared to Q1, Q3, or Q4 ($p<0.01$). Probe learning index in Fig. 3 and Fig. 4 is calculated from probe trial trajectories as the amount of time during the first 60 seconds after leaving the starting quadrant that flies spent searching in Q2 (the quadrant where they have been trained to locate the cool tile) minus the amount of time spent searching in Q4 (a quadrant that is the same distance from the starting quadrant, but the wrong direction) divided by the total time in both quadrants. p-values reported in Fig. 3c legend were calculated using a one-tailed t-test. Probe learning index scores in Fig. 3d were tested for statistical significance ($p<0.05$) using one-way ANOVA with a Bonferroni correction for multiple comparisons and pairwise comparisons to the uncoupled condition in Fig. 3c. Probe learning scores are significantly greater than uncoupled for up to 120 minutes after training. We note that although the Place learning index is not significantly different from the uncoupled control at 4 and 6 hours, they are significantly greater than 0 (one-tailed t-test, $p<0.05$). Since flies were left in the arena between training and testing (up to 8 hrs), *control* refers to siblings placed in the arena for an equivalent window of time (i.e. 8 hours) prior to training followed by

immediate testing. p-values reported in Fig. 4 legend were calculated using a one-tailed t-test comparing place learning scores before (white box) and after (grey box) Kir2.1 induction. Probe learning index scores reported in Fig. 3c, and Fig. 4g were also tested for statistical significance using one-way ANOVA with a Bonferroni correction for multiple comparisons. In Fig. 3c, flies trained in the coupled condition show significantly higher probe learning scores ($p<0.01$) when compared to flies trained in the uncoupled or dark conditions. There is no significant difference (at $p<0.05$ level) between uncoupled and dark. In Fig. 4g, R15B07 and R28D01 flies shifted to $30^{o}C$ are significant at $p<0.01$ when compared to control flies. No other comparisons to control flies are significant at $p<0.05$. In all box and whisker plots, the "whiskers" cover the range of the data, excluding outliers. Outliers are defined as data points greater than the 75th percentile of all data points plus 1.5 times the interquartile range ($q_3 + w(q_3 - q_1)$) or data points less than the 25th percentile of all data minus 1.5 times the interquartile range ($q_1 - w(q_3 - q_1)$) . The majority of datasets presented as box plots contain no outlying data.

Following the probe trial, flies were tested for thermal preference by setting alternating tiles on the TEM array to either $25^{o}C$ or $36^{o}C$. Flies were allowed to distribute for 2 minutes before the cool and warm tiles were switched. The flies were then allowed another 2 minutes to re-distribute and the thermal aversion index was calculated as the amount of time flies spent at $25^{o}C$ minus the amount of time spent at $36^{o}C$ divided by the total time. Finally, flies were tested for normal optomotor responses by rotating a checkerboard pattern on the visual panorama clockwise and then counterclockwise at 90° per second for 45 seconds. Optomotor responses are reported as the mean rotational velocity (in the direction of the stimulus) of the flies over the course of these trials. No significant differences are observed in thermal aversion or optomotor

response at p<0.05 using one-way ANOVA with a Bonferroni correction for multiple comparisons.

Non-visual place learning

Work from the Heisenberg[15, 24, 35], Zars[25, 36, 37], and Strauss[17] groups have shown that flies can use idiothetic cues and path integration to navigate (in several forms of non-visual place memory tasks). To address whether idiothetic (i.e. non-visual) cues are sufficient to guide navigation in the thermal-visual arena we tested flies using a set of modified training protocols that included (a) keeping the cool tile stationary, (b) rotating the cool tile in a constant direction, and (c) randomly rotating the cool tile between trials, all in the dark. Next, we tested flies with (d) a stationary visual panorama and a randomly relocated cool tile, (e) with a randomly rotating visual panorama and a stationary cool tile, and (f) with random, and independent relocations of the visual panorama and cool tile (Supplementary Fig. 8). We see no evidence of place learning when flies are trained and tested with any of these modified protocols. We note that flies may use idiothetic information while navigating; however, this experience is not sufficient for the formation of a place memory. In order to disperse flies from the stationary cool tile between trials the entire array was heated to 36ºC for 60s prior to the start of each trial. When trained with the standard coupled visual panorama (see Fig. 1 and 2), this manipulation does not impair visual place learning (data not shown).

Olfactory conditioning

Olfactory conditioning experiments were based on experiments using an elevated T-maze as described in Tully and Quinn[23]. The conditioning protocol was modified to use temperature as

the unconditioned stimulus rather than electric shock (Fig. 4m, Supplementary Fig. 6). During conditioning, the training tube was heated to 36°C concurrent with delivery of the first odor by passing a 5V, 0.43A current through a custom built insulated resistance wire mesh (Pelican Wire, 29 AWG Nichrome 60 w/Kapton) inserted into the training tube. Odors were delivered by bubbling an air stream through a vial containing odorant diluted in paraffin oil. Odors used were 5% 4-methylcyclohexanol (MCH), flow rate 128ml/min and 5% 3-octanol (OCT), flow rate 60 ml/min. Flow rate through training and testing tubes was normalized to 800ml/min by combining the odorant stream with a humidified clean air stream. ~200 flies were tested in each experiment, ½ conditioned to MCH, ½ conditioned to OCT. Learning indexes were calculated as the average learning index of the two groups. All mushroom body lines (R9A11, R10B08, and R67B04) are significantly impaired in olfactory learning when compared to control flies ($p<0.05$ using one-way ANOVA with a Bonferroni correction for multiple comparisons). No eb lines are significantly different from control.

Tethered flight experiments

Closed loop tethered flight experiments were performed as previously described using a cylindrical LED display and an optical wing beat analyzer to measure fly responses[29]. To test whether flies were capable of discriminating the visual features of the panoramic pattern in the visual place learning arena, we examined the orientation preference of flying flies for a flight arena pattern that was composed of 4 quadrants that display 15° wide bar gratings, in either a vertical (quadrants 1, 3) or horizontal (quadrants 2, 4) direction. Each fly was allowed to selectively orient under behavioral closed loop with this pattern for 5 trials of 50 seconds each, as part of an experimental series consisting of other closed and open loop trials, for which no

further data is shown. Flies showed a clear preference for the vertical bars, and so we quantified the behavior with an orientation index that was calculated as the amount of time flies oriented towards vertical bars minus the amount of time orienting towards horizontal bars divided by the total time. No significant differences are observed in the orientation index at $p<0.05$ using one-way ANOVA with a Bonferroni correction for multiple comparisons.

Experimental animals

All flies used were female and, unless otherwise noted, are DL wildtype. This strain is a laboratory culture descended from interbreeding dozens of wild caught isofemale lines, established in 1995 and maintained by Michael Dickinson's laboratory[38]. Flies were reared on standard media at 25°C on a 16hr light/8hr dark cycle. Visual place learning experiments were performed with 4 day old adult flies during hours 11-15 of the flies' subjective day (where hour 0 corresponds to the transition from dark to light) in a room kept at 25°C and 40% RH. For neural silencing experiments, w+; tubP-GAL80^{ts}; UAS-Kir2.1 flies (backcrossed 10 generations into DL wildtype genetic background to control for the effects of genetic background[39] and known behavioral deficits with flies homozygous for w1118[37]) were crossed to GAL4 driver lines and reared at 18°C. Two day old adult females were temperature shifted to 30°C for 40 hours and then returned to 18°C for 2 hours prior to testing. GAL4 driver lines were constructed as described in Pfeiffer et al.[40] and provided by Gerry Rubin. Control flies are w^{1118}; attP2 (the same genetic background as the GAL4 lines) crossed to w+; tubP-GAL80^{ts}; UAS-Kir2.1. To ensure backcrossing into the DL genetic background does not create conditions for PM hybrid dysgenesis (i.e. mobilization of p-elements), our effectors/reporters (all marked with mini-white) are kept over balancer chromosomes and we regularly monitor for the appearance of the reporter

in the wrong chromosome (i.e. indicative of transposition). Over the 3 years that we have been

crossing and monitoring these stocks, we see no evidence of transposition.

Supplementary References

31.   Martin, J.R., Raabe, T. & Heisenberg, M. Central complex substructures are required for the maintenance of locomotor activity in Drosophila melanogaster. *J Comp Physiol A* **185**, 277-288 (1999).
32.   Sayeed, O. & Benzer, S. Behavioral genetics of thermosensation and hygrosensation in Drosophila. *Proc Natl Acad Sci USA* **93**, 6079-6084 (1996).
33.   Zars, T. Two thermosensors in Drosophila have different behavioral functions. *J Comp Physiol A* **187**, 235-242 (2001).
34.   Strauss, R., Schuster, S. & Gotz, K.G. Processing of artificial visual feedback in the walking fruit fly Drosophila melanogaster. *J Exp Biol* **200**, 1281-1296 (1997).
35.   Wustmann, G. & Heisenberg, M. Behavioral manipulation of retrieval in a spatial memory task for Drosophila melanogaster. *Learn Memory* **4**, 328-336 (1997).
36.   Zars, T., Wolf, R., Davis, R. & Heisenberg, M. Tissue-specific expression of a type I adenylyl cyclase rescues the rutabaga mutant memory defect: in search of the engram. *Learn Mem* **7**, 18-31 (2000).
37.   Diegelmann, S., Zars, M. & Zars, T. Genetic dissociation of acquisition and memory strength in the heat-box spatial learning paradigm in Drosophila. *Learn Memory* **13**, 72-83 (2006).
38.   Dickinson, M.H. Haltere-mediated equilibrium reflexes of the fruit fly, Drosophila melanogaster. *Philos T Roy Soc B* **354**, 903-916 (1999).
39.   de Belle, J.S. & Heisenberg, M. Expression of Drosophila mushroom body mutations in alternative genetic backgrounds: a case study of the mushroom body miniature gene (mbm). *Proc Natl Acad Sci USA* **93**, 9875-9880 (1996).
40.   Pfeiffer, B.D.*, et al.* Tools for neuroanatomy and neurogenetics in Drosophila. *Proc Natl Acad Sci USA* **105**, 9715-9720 (2008).